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CHOLINERGIC NEUROTRANSMISSION IN THE  
MAMMALIAN RETINA

ANNUAL SUMMARY REPORT

Roberta G. Pourcho

November 30, 1984

Supported by  
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21701-5012

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Wayne State University  
Detroit, Michigan 48202

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appear to play a major role in cholinergic or related pathways and may be particularly susceptible to organophosphate poisoning.

The biochemical component of the study has centered on the development of a superfusion system in which to monitor the release of various amino acid transmitters in response to application of acetylcholine. Preliminary experiments suggest that cholinergic amacrine cells are presynaptic to glycinergic cells in the cat retina. After the normal pattern has been established, it should be possible to investigate the effects of changes in the level of acetylcholinesterase on these responses. (R.D.)

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## SUMMARY

This study seeks to determine the cellular localization of acetylcholine and acetylcholinesterase in the cat retina, the interactions of these compounds with each other and with other neuroactive substances, and the effects of anticholinesterase organophosphates on normal retinal function. Cytochemical studies are being employed to identify the neurons which participate in cholinergic neurotransmission while biochemical methods are being developed to investigate the interactions of acetylcholine and acetylcholinesterase.

Among the cytochemical studies conducted to date are localization of those neurons which are capable of synthesis of (3H)acetylcholine from a (3H)choline precursor and identification of the cells which produce the acetylcholine destructive enzyme, acetylcholinesterase. We have also carried out experiments with AF64A, a toxin thought to selectively destroy cholinergic neurons.

In other mammalian retinas, cholinergic neurons appear to be a rather consistent population of amacrine and displaced amacrine cells with a mirror-symmetrical distribution in strata 2 and 4 of the inner plexiform layer. This cell type has been characterized most extensively in the rabbit retina where it has a dense starburst-like dendritic tree. Morphological studies in our laboratory have shown that the cat retina contains a similar subpopulation of amacrine and displaced amacrine cells but with a much more sparse dendritic pattern. If cholinergic, the sparse dendrites of these cells would be difficult to visualize by methods with limited resolution such as the freeze-dry autoradiography which was used to identify cholinergic cells in the rabbit retina.

As expected, freeze-dry autoradiography of (3H)acetylcholine in cat retina provided no evidence of labeling in amacrine and displaced amacrine subpopulations. However, this procedure did result in the labeling of many ganglion cells. Autoradiography of cat retina in which phosphomolybdic acid was used to retain (3H)acetylcholine showed labeling of amacrine and displaced amacrine cells in addition to a number of ganglion cells. Cytochemical studies demonstrated the presence of acetylcholinesterase activity in a few amacrine cells and in essentially all of the cells the ganglion cell layer. Furthermore, the ganglion cells appeared to be susceptible to destruction by the cholinotoxin, AF64A.

The cytochemical findings to date suggest that cat starburst amacrine cells, like their more highly developed

counterparts in other mammalian retinas, are cholinergic. The available physiological data suggests that these neurons are responsible for the intraretinal effects of acetylcholine. In addition, ganglion cells were found to be active in the metabolism of (3H)choline, to contain acetylcholinesterase, and to be highly susceptible to morphological changes induced by a presumptive cholinotoxin. These findings should provide a basis for future investigations of the cells involved in cholinergic mechanisms in the retina.

The biochemical component of this project has centered on the development of an appropriate methodology for assessing the effects of acetylcholine on the release of other neurotransmitters by a superfused cat retina. The superfusion system allows for continuous flow of warm, oxygenated buffer over the tissue with intermittent addition of acetylcholine. The superfusate is collected in 1 ml aliquots and analyzed for amino acid content by high performance liquid chromatography. Preliminary findings indicate that acetylcholine produces no significant changes in the release of gamma-aminobutyric acid or taurine but causes a pronounced increase in glycine efflux. We plan to obtain full quantitation of these results and extend the studies to include assays of the release of acetylcholine in response to application of other retinal transmitters.



## FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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#### STATEMENT OF PROBLEM:

Acetylcholine (ACh)\* is a classical neurotransmitter which participates not only in the control of muscle contraction but also performs numerous other essential functions within the central nervous system. These functions are at risk when exposure to organophosphate compounds occurs. Among the active organophosphates are several chemical agents considered for military use. This study is directed toward an understanding of how ACh functions in a particular region, the retina; how it interacts with other retinal neurotransmitters; and how these functions are influenced by acetylcholinesterase (AChE) and by exposure to anticholinesterase organophosphates.

#### BACKGROUND:

The synthesis of ACh is mediated by the enzyme choline acetyltransferase (ChAT) while its destruction is mediated by AChE. All of these compounds are present with regional variations in concentration throughout the central nervous system (CNS). The retina is no exception and its ready accessibility and laminar organization make it a particularly useful model in which to study cholinergic interactions. Vertebrate retinas consist of five major classes of neuron with photoreceptor cells, both rods and cones, receiving the light stimulus and transmitting a signal to bipolar cells which, in turn, relay the message to ganglion cells which project to the brain. The message is modified in the outer plexiform layer (OPL) by horizontal cells and in the inner plexiform layer (IPL) by amacrine cells. Biochemical analysis has shown that ACh and its related enzymes are concentrated in the inner layers of the retina where amacrine and bipolar cells are the presynaptic neurons.

The seemingly simple organizational pattern of the retina is made more complex by the presence of numerous subpopulations of neurons within the five major classes. In the cat retina, there are more than twenty morphologically distinct subpopulations of both amacrine and ganglion cells (1). The existence of comprehensive anatomical classifications as well as numerous physiological and pharmacological studies makes the cat retina a particularly useful model in which to study the details of neurotransmission. Furthermore, an increasing volume of data from this and other laboratories is accumulating regarding the identification of the transmitters employed by specific subclasses of neurons (2,3,4).

The specific cells in the cat retina which utilize ACh as a transmitter have not been identified previously. In rabbit retina, Masland and Mills (5) have identified two

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\*Abbreviations are defined in the glossary on p. 23.

subpopulations of cells which exhibit the capacity to synthesize (3H)ACh from (3H)choline. These include a subpopulation of amacrine cells with cell bodies located in the inner nuclear layer (INL) and a second subpopulation of cells which are morphologically amacrine cells but whose cell bodies are displaced to the ganglion cell layer (GCL). These neurons ramify in strata S2 and S4, respectively, of the IPL and exhibit a characteristic radial symmetry leading to their designation as starburst amacrines (6). Although there is pharmacological evidence that the cat retina also contains cholinergic cells, the lower levels of ACh as well as those of AChE and of ChAT (7) suggest that there are fewer cholinergic cells or a reduced activity level of a comparable population.

A role for ACh as a transmitter in the cat retina was indicated in studies by Straschill (8) showing that ACh increases the spontaneous activity of certain ON-type ganglion cells. More recently, Ikeda and Sheardown (9) demonstrated that ACh causes an enhanced response in ganglion cells which produce a transient activity.

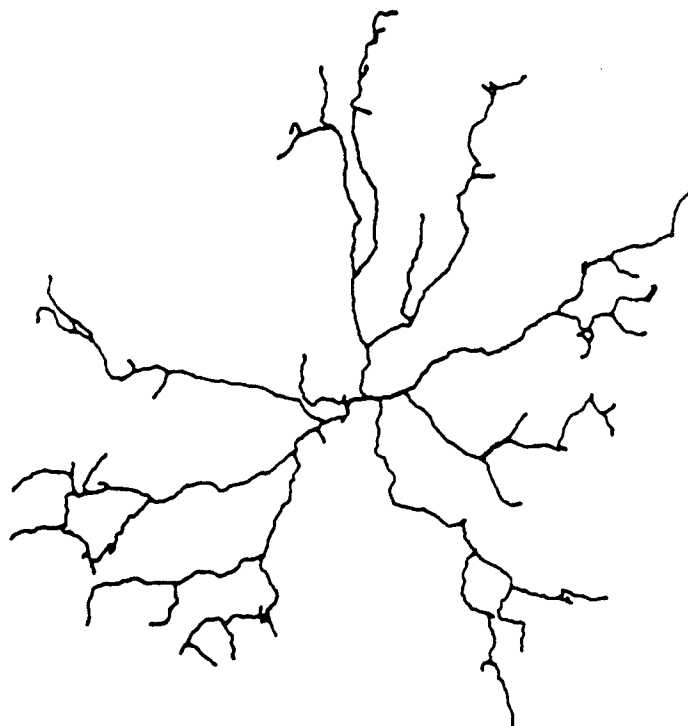


Fig. 1. Computer-generated drawing of starburst-like amacrine cell from cat retina. This cell was typical of several found in Golgi-impregnated cat retinas. The radially symmetrical branching pattern is characteristic although the dendritic tree is much less dense than that in the rabbit. This cell was displaced to the ganglion cell layer.

Morphological studies in our laboratory employing Golgi impregnation techniques have shown that the cat retina contains a subpopulation of amacrine and displaced amacrine cells with features similar to those of the rabbit starburst amacrine (Fig. 1). Like the cholinergic cells of the rabbit, these cells ramify in S2 and S4 of the IPL. However, their dendrites are more sparse than those seen in the rabbit.

#### APPROACH:

The goal of these studies is to investigate the cholinergic system in the cat retina, making use of the existing knowledge of the anatomy and neurochemistry of this retina to help elucidate the interactions of ACh and AChE and the effects of these substances on other neurotransmitter systems. A thorough understanding of the cholinergic system in a well-defined system such as the retina should help to establish a basis for reducing the effects of exposure to anticholinesterase drugs.

These studies involve two lines of investigation, each related to the cholinergic system in a mammalian (cat) retina. Cytochemical methods are being used to provide morphological identification of cholinergic neurons and AChE-containing neurons while biochemical methods are being developed to investigate the relationships of cholinergic neurons with those cells to which they are pre- or post-synaptic. The cytochemical studies involve both light and electron microscopy and will provide the opportunity for comparison of data with ongoing studies of other neurotransmitters in the cat retina. The biochemical studies employ high performance liquid chromatography (HPLC) to assay the release of transmitter substances by a superfused retina in response to challenge by other neurotransmitters.

#### CYTOCHEMICAL STUDIES

##### (1). Autoradiographic localization of (3H)ACh.

Materials and methods. Freshly dissected retinas were processed for (3H)ACh autoradiography following the methodology of Masland and Mills (5). Tissue was incubated for 15 min in a balanced salt solution (119.6 mM NaCl, 3.5 mM KCl, 1.15 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 0.1 mM  $\text{KH}_2\text{PO}_4$ , 22.6 mM  $\text{NaHCO}_3$ , 0.4 mM  $\text{Na}_2\text{HPO}_4$ , and 10.0 mM glucose) containing 0.3  $\mu\text{M}$  (3H)choline. After the incubation, the tissue was washed in an excess of unlabeled choline in a similar buffer but with 20 mM  $\text{MgSO}_4$  and 0.2 mM  $\text{CaCl}_2$ , conditions designed to favor the retention of (3H)ACh in the tissue. Since ACh is normally water soluble, special care was exercised to prevent loss during processing for autoradiography. Some retinas were freeze-dried, fixed with osmium vapor, embedded in Epon-Araldite and sectioned for dry autoradiography as described by Masland and Mills (5). Sections 1  $\mu\text{m}$  thick were placed on glass microscope slides and covered with coverslips previously dipped into Ilford K-5

emulsion and allowed to dry. The sections were exposed for 6-8 weeks and the autoradiography was developed. Other retinas were fixed by immersion in 3% glutaraldehyde with 2% phosphomolybdic acid (PMA), postfixed in 2% osmium tetroxide also containing 2% PMA and then processed routinely for autoradiography. This procedure was adapted from Tsuji (10) who demonstrated that PMA serves to retain ACh in tissue during processing through aqueous solvents.

**Results.** Rabbit retinas which were processed for freeze-dry autoradiography showed labeling in amacrine and displaced amacrine cells as well as in distinct bands within the IPL, confirming the findings of Masland and Mills (5) and showing that the procedure was being carried out properly in our laboratory (Fig. 2). Cat retinas, processed similarly, showed labeling in numerous cells in the ganglion cell layer, including large cells which could be identified as ganglion rather than displaced amacrine cells (Fig. 3). The cat retinas which were fixed in PMA and then processed for (3H)ACh autoradiography showed labeling in amacrine and displaced amacrine cells and in many, if not all, ganglion cells (Fig. 4).



Fig. 2. Freeze-dry autoradiograph of rabbit retina, incubated with (3H)choline for synthesis of (3H)ACh. Two distinct bands of label can be seen in the inner plexiform layer. Cell bodies of displaced amacrine cells (arrows) in the GCL are also visible. This preparation typically shows labeled amacrine cells also, although none are visible in this particular section. OS, outer segments; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 10  $\mu$ m.



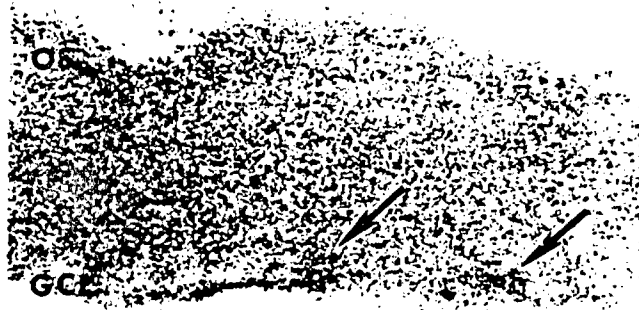


Fig. 3. Freeze-dry autoradiograph of cat retina prepared to show newly synthesized (3H)ACh. Label is seen in two large cells in the GCL (arrows). The labeling in the outer retina is probably due to synthesis of (3H)phosphatidylcholine. OS, outer segments; GCL, ganglion cell layer. Bar, 10  $\mu$ m.



Fig. 4. Autoradiograph of cat retina, incubated with (3H)choline for synthesis of (3H)ACh, then fixed in phosphomolybdic acid to retain the (3H)ACh. Heavy labeling is seen in an amacrine cell (A), a ganglion cell (G), and a smaller cell in the ganglion cell layer which is likely a displaced amacrine cell (dA). The labeling in the outer segments (OS) is indicative of the formation of (3H)phosphatidylcholine. Bar, 10  $\mu$ m.

Discussion. The labeling of amacrine and displaced amacrine cell bodies with (3H)ACh as shown in Fig. 4 is consistent with the possibility that in cat, as in rabbit, the cholinergic cells include both amacrine and displaced amacrine cells. The labeling of ganglion cells raises the question as to whether these cells also might be cholinergic. However, there is little supporting evidence for this interpretation. In their freeze-dry autoradiographic study of rabbit retina, Masland and Mills (5) also observed a labeling of ganglion cells and hypothesized that it might be due to an increased incorporation of (3H)choline into membrane components of these neurons because of their large size. Further study will be required to resolve this issue.

(2). Identification of putative cholinergic neurons using the cholinotoxin AF64A.

Materials and Methods. Adult cats and rabbits were injected intravitreally with 50 nmol AF64A and allowed to survive for 1-3 days before sacrifice and removal of their retinas. This toxin is the activated aziridinium ion form of a choline mustard. It is postulated to be preferentially destructive of cholinergic neurons because of their high affinity choline uptake (11). Tissue was processed for light and electron microscopy and analyzed for morphological evidence of neurotoxicity. In order to provide confirmation of an effect on the cholinergic system, some specimens were processed for cytochemical or biochemical determination of AChE. The cytochemical localization of AChE was carried out by the method of VanOoteghem and Shipley (12) and the biochemical quantitation of AChE by the method of Ellman et al. (13)

Results. Rabbit retinas showed a selective destruction of cell bodies in both amacrine and ganglion cell layers. After 3 days of exposure to the neurotoxin, a number of degenerating cells could be seen in the amacrine portion of the IPL and a lesser number in the ganglion cell layer. There was a considerable degree of vacuolation in the ganglion cell layer. Cat retinas showed extensive degeneration of neurons in the ganglion cell layer at all times observed (Fig. 5) and an apparent destruction of nearly all cells in this layer within 3 days. An invasion of neutrophils was seen frequently in both AF64A injected eyes and in control eyes which received an injection of saline and so appeared to be unrelated to the toxic effects of AF64A. Both enzyme cytochemistry and biochemical assay indicated that the levels of AChE activity in both species were reduced after exposure to AF64A (Table 1).

Discussion. Application of AF64A in chicken retina (11) is reported to cause selective destruction of the cholinergic neurons. Our findings in rabbit retina are consistent with the destruction of cholinergic starburst amacrine and displaced amacrine cells, assuming that the AF64A had access first to the displaced amacrine cells and later to



Fig. 5. Electron micrograph of cat retinal ganglion cell showing extensive degeneration 2 days after intravitreal injection of 50 nmol AF64A. The ganglion cell (G) shows cytoplasmic vacuolation which extends throughout its dendrites. The optic fiber layer is severely vacuolated, with large open areas and invasion of neutrophils (N). Bar, 1  $\mu$ m.

Table I

Acetylcholinesterase activity in retinas injected with  
50 nmole AF64A (2 day survival)

	<u>AF64A-treated</u>	<u>Control</u>	<u>% Change from control</u>
Cat	$6.45 \times 10^{-5}$	$11.08 \times 10^{-5}$	-41.8%
Rabbit	$9.06 \times 10^{-5}$	$13.48 \times 10^{-5}$	-32.6%

the conventional amacrine cells deeper in the retina. However, cat retinas showed extensive degeneration of neurons in the ganglion cell layer at nearly all times observed (Fig. 5) and an apparent destruction of ganglion cells within 3 days. The reduction in AChE activity indicates a major effect on the cholinergic system.

(3). Cytochemical localization of AChE in normal and diisopropylfluorophosphate-treated cat retinas.

Materials and Methods. Freshly dissected cat and rabbit retinas were processed for cytochemical localization of AChE using the modification of the Koelle technique developed by VanOoteghem and Shipley (12). Briefly, this method employs an incubation medium containing 2 mM copper sulfate, 10 mM glycine, 50 mM sodium acetate, and 4.2 mM acetylthiocholine iodide. Incubations were carried out at 37°C for 45-90 minutes with gentle agitation. Although initially developed as a light microscopic technique, we have found that this methodology is also applicable for electron microscopy. In order to facilitate visualization of AChE in neuronal cell bodies, some cats were anesthetized and injected with diisopropylfluorophosphate (DFP) [1.2-3.0 mg/kg] and then maintained for 12 hours to permit newly synthesized AChE to accumulate. These animals were then overdosed with pentobarbital and the retinas removed for AChE cytochemistry.

Results. Cat retinas processed for AChE localization showed reaction product throughout the IPL. The distribution was similar to that observed in rabbit retinas although much less enzymatic activity is present in the cat, as evidenced by the longer periods of time required to achieve satisfactory levels of reaction product. DFP-treated retinas showed the presence of newly synthesized AChE in cell bodies of a relatively small number of amacrine cells (Fig. 6) and in essentially all of the cells in the ganglion cell layer (Fig. 7). The amount of reaction product was greatly reduced when BW284, a specific inhibitor of AChE, was added to the incubation medium. After seeing the large amounts of AChE in ganglion cell bodies following DFP treatment, we were able to detect it, in smaller amounts, in control retinas processed without DFP.

Discussion. Significant amounts of AChE were found in



Fig. 6. Flat mount preparation of cat retina, 12 hours after DFP treatment, showing synthesis of AChE by amacrine cells (arrows). The piece of retina is slightly uneven so that ganglion cells are visible at the top of the field. Bar, 50  $\mu$ m.



Fig. 7. Same retina as in Fig. 6 but with the focus at the level of the ganglion cells. Essentially all of the ganglion cells appear to be producing AChE. A blood vessel can be seen coursing across the field. Bar 50  $\mu$ m.

all ganglion cells of the cat retina. The amount of AChE in these cells was increased following treatment with DFP, apparently in response to destruction of existing stores of the enzyme. The presence of AChE in ganglion cells is consistent with the observations of Illing and Graybiel (14). Other investigators have reported the presence of AChE in embryonic ganglion cells in several species. Although AChE cannot be considered a reliable marker for cholinergic neurons, the observation that all of the ganglion cells produce AChE shows the need for further investigation.

#### BIOCHEMICAL STUDIES

Materials and Methods. Freshly dissected cat retinas were maintained in a warm, oxygenated environment in the balanced salt solution developed by Ames and his colleagues for mammalian retinas (5). The incubation medium is continuously changed and the efflux is collected in 1 ml aliquots for subsequent analysis by HPLC. Amino acids are derivitized with o-phthalaldehyde and separated on a Waters Resolve column using a low salt, high organic buffer. Samples are monitored with a fluorescence detector. Methods are being developed for assaying the amount of small peptides using a C18 Bondapak column and monitoring the samples at 280 nm. This methodology will be useful in determining whether changes in AChE levels influence neuroactive peptides in the retina.

Results. Preliminary experiments were conducted to determine the washout curve for endogenous amino acid efflux. After determining that 20 minutes is an appropriate time interval, we have applied varying concentrations of ACh for a 2 minute interval and monitored samples for changes in rate of efflux of gamma-aminobutyric acid, glycine, and taurine. Concentrations of ACh less than 1 mmol were ineffective in causing amino acid release. After exposure of retinas to 1 mol ACh, the rate of glycine efflux was increased while the rates of release of gamma-aminobutyric acid and taurine appeared to be essentially unchanged.

Discussion. If these results are confirmed, they will suggest that cholinergic amacrine cells may synapse on glycinergic neurons in the cat retina. However, these methods will not provide information as to which of the several subclasses of putative glycinergic neurons might be affected.

#### CONCLUSIONS:

The main finding during the year covered in this report is that amacrine and displaced amacrine cells in the cat retina have the capacity to synthesize ACh, while the ACh-hydrolyzing enzyme, AChE, is present not only in amacrine cells but also in essentially all of the ganglion cells. Both amacrine and ganglion cells appear to be susceptible to the presumed cholinotoxin AF64A. These findings provide a basis for use of the cat retina as a model system for the study of cholinergic interactions in the central nervous system.

#### RECOMMENDATIONS:

It will be important to continue cytochemical localizations of cholinergic markers in order to more precisely define the location of cholinergic and cholinceptive cells in the retina. The use of an antiserum to choline acetyltransferase should be useful for confirming the identity of cholinergic cells. Attention should also be directed to the localization of muscarinic and nicotinic ACh receptors in the retina and the localization of the sites of action of anti-AChE organophosphates.

AF64A appears to produce extensive destruction in the cat retina. These effects should be studied in greater detail, particularly with regard to selectivity or non-selectivity of the toxic effects. It would be of interest to determine not only the changes in ACh and AChE levels following AF64A treatment but also the changes in other neurotransmitters.

HPLC experiments should include a definitive study of the effect of ACh on the release of other transmitters and, conversely, the effects of other transmitters on the release of ACh. After these parameters have been established, it should be possible to investigate the effects of AChE and anti-AChE compounds on normal release patterns.





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## GLOSSARY

ACh	Acetylcholine
AChE	Acetylcholinesterase
AF64A	Azridinium ion of choline mustard
ChAT	Choline acetyltransferase
CNS	Central nervous system
DFP	Diisopropylfluorophosphate
GABA	Gamma-aminobutyric acid
GCL	Ganglion cell layer of retina
(3H)ACh	Tritiated acetylcholine
HPLC	High performance liquid chromatography
INL	Inner nuclear layer of retina
IPL	Inner plexiform layer of retina
OPL	Outer plexiform layer of retina
PMA	Phosphomolybdic acid
S2, S4	Strata of retinal inner plexiform layer



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